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SOME OBSERVATIONS ON THE NON-SPECIFICALLY
ENHANCED RESISTANCE OF MICE AGAINST
INTRAPERITONEAL INFECTION
WITH *SALMONELLA ABORTUS-EQUI*

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Mouse-protection test is very practical for assaying the potency of various vaccines. In this test, administrations of the immunizing injections and of the challenge doses are often made by the intraperitoneal route. However, the non-specifically enhanced resistance of mice that follows the intraperitoneal injection of various substances has been observed by many workers. This phenomenon may be an important source of errors in all mouse-protection tests in which the challenge dose is injected by the intraperitoneal route.

FELIX pointed out that many workers engaged in experiments on typhoid immunity use the faulty method of injecting both immunizing and challenge doses by the intraperitoneal route.

The present authors have studied on the mice-protection tests of the killed vaccine of *S. abortus-equi*. In a previous paper, HIRATO et al. recognized the high potency of this vaccine in the case of injecting both the immunizing and the challenge dose by intraperitoneal route. However, it was left as an unsolved problem whether the non-specific protective mechanisms would play some part in this protection test, and the authors stated that some additional experiments are needed for the establishment of this point.

ØRSKOV and KAUFFMANN, PHILIPSON and ØRSKOV examined in detail the degree and duration of this kind of non-specific resistance. From those studies, it is learned that one intraperitoneal dose of a vaccine of some kinds of bacteria induces in mice a high degree of immunity to intraperitoneal challenge with the other kinds of bacteria and that the repeated injections of the vaccine induce a longer duration of the same type of immunity.

From these well established facts, it would seem that the same thing might interfere with the results of the mouse-protection test of equine paratyphoid vaccine. The present study was carried out to

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ascertain the non-specific rise of resistance in case of assaying the protective value of this vaccine.

MATERIALS AND METHODS

Bacterial Strain: The strain of *S. abortus-equi* used for the preparation of vaccine and the test culture was a pretty fresh one. A relatively old stock culture of *Escherichia coli* var. *communior* was used for preparation of a heterologous vaccine. It had no common antigenic constituents with *S. abortus-equi*.

Vaccine Preparation: Heat-killed vaccine was prepared from saline suspension of plain nutrient agar culture by heating at 60°C for 40~50 minutes.

Chrome-vaccine: Bacterial suspension (1% formol saline) was mixed with equal volume of 0.4% alum chromate solution. After having stood for 2 days at room temperature, this suspension was centrifuged and the sediment was washed two times in normal saline.

Mouse-test: The same strain of white mouse was used throughout as described in previous papers on the mouse-protection test of equine paratyphoid vaccine. They were of female sex in almost all experiments, and weighed about 15 g at the time of infection. Mice were immunized by intraperitoneal route, with 0.5 ml saline suspension containing respectively each vaccine dose of homologous or heterologous bacteria and the challenge dose of *S. abortus-equi* was given in the same manner after various intervals. In order to avoid any fluctuation of bacterial number of the challenge dose, the infection was made simultaneously against all groups of mice having different intervals after the immunizing injections. Heart blood of mice which died was cultured to ascertain whether death had occurred as a result of infection. In the latter part of the experiments, bacilli in tail blood of infected mice were examined by cultural method. Two drops of blood were cultured on ENDO agar and a loopful of blood was enriched with broth containing saponine and sodium citrate. Other detailed descriptions of experimental methods will be given in the next chapter.

EXPERIMENTAL RESULTS

1. The Non-specific Resistance of Mice to Fatal Infection with a Large Dose of *S. abortus-equi*

In the mouse-protection test of equine paratyphoid vaccine, the challenge dose is given after an interval of about 10 days with a sufficient dose usually to cause septicaemic death.

Firstly to investigate the effect of non-specifically increased intraperitoneal resistance, the authors used two kinds of killed vaccine. Each group of 3~4 mice was injected intraperitoneally with 0.2 mg of heat-killed bacilli of abortion or coli at periods ranging from 1 to 14 days before infection as indicated in table 1. Intraperitoneal challenge dose of 0.15 mg of abortion bacilli was given to each immunized group simultaneously.

TABLE 1. *Survival Rate of Mice Immunized with Heterologous or Homologous Vaccines within 30 Days after Fatal Infection with S. abortus-equi*

GROUPS IMMUNIZED WITH HEAT-KILLED BACILLI OF	INTERVALS BETWEEN VACCINATION AND INFECTION						
	1	2	4	8	10	14 days	
<i>S. abortus-equi</i>	0/4	1/4	1/4	4/4	3/3	3/3	—
<i>E. coli</i>	2/4	0/4	0/4	0/4	0/3	0/3	—
Controls	—	—	—	—	—	—	0/4

Vaccine dose: 0.2 mg i. p.

Challenge dose: 0.15 mg i. p.

A glance at table 1 shows that a certain protective resistance appeared in a mice group infected at an interval of 1 day after heterologous vaccination. In case of homologous vaccination, the mice group infected at a 2- and 4-day interval indicated low survival rate. This result seems to indicate that mice acquire some resistance against fatal infection when they are immunized with heterologous or homologous vaccine and these non-specific intraperitoneal resistances appear at an earlier period in comparison with specific immunity.

In the latter experiment, a larger dose of vaccine was used than that in the previous experiment, and infected mice were observed clinically for 30 days after infection. This was done because, in natural condition, mice infected with abortion

TABLE 2. *Resistance of Mice at Various Intervals after Intraperitoneal Treatment to Infection with a Large Challenge Dose of S. abortus-equi*

INTERVALS BETWEEN VACCINATION AND INFECTION (Days)	SURVIVAL DAYS OF MICE IMMUNIZED WITH HEAT-KILLED BACILLI OF		
	<i>S. abortus-equi</i>	<i>E. coli</i>	
1/3	3, 4, 4, 5, 5.	5, 5, 5, 5.	
1	4, 4, 5.	6, 6, 6, s.	
4	—	5, 6, 6, 7.	
7	—	1, 4, 4, 5.	
10	S, S, S.	4, 4, 5, 7.	
"	—	* 4, 5, 6, s.	
"	—	** 4, 5, S, S.	
Controls	—	—	1, 5, 6, 8,

Vaccine dose: 0.5 mg i. p.

* This group was given 2 doses of vaccine (0.2, 0.3) with a 7-day interval.

** This group was injected with 2 doses of vaccine (0.5, 0.5) with a 7-day interval.

Challenge dose: 0.15 mg i. p.

S: Survived and healthy for 30 days after infection.

s: Survived but suffered from septicaemia.

bacilli regularly get septicaemia, but they do not always die. Therefore by observing the survival rate only, one is apt not to learn whether they are perfectly protected from septicaemia or whether they escaped from the danger of septicaemic death. Septicaemic mice show clinical signs such as hair roughening, "arched" back or loss of appetite and are easily discriminated from healthy mice.

The data in table 2 seem to establish that 0.5 mg of heterologous vaccine scarcely produced non-specific resistance. However, it should be noticed that half the number of mice survived in a group which were immunized with a dose of 1 mg coli vaccine and infected 10 days later. Further, these survived mice did not suffer from both the primary and secondary septicaemia similarly to mice homologously vaccinated.

From the above observations, it may be stated that mice immunized with killed vaccine of abortion or coli bacilli resist slightly against fatal infection with a large dose such as 0.15 mg of abortion bacilli at the early period after vaccination, but 1 mg of hetero-vaccine gives a complete protective power to some of the mice on the 10th day after vaccination.

2. Effect of the Non-specific Resistance of Mice on the Destruction of Intraperitoneally Injected Bacilli

The previous experiments seem to show that most of the mice treated specifically or non-specifically do not strongly demonstrate non-specific resistance to fatal infection with a large dose of abortion bacilli. However, it is probable that severe infection caused by a large challenge dose may cover the feeble rise in non-specific resistance, so how to detect such a feeble resistance should be considered. According to the previous investigations, abortion bacilli injected with a proper dose into the peritoneal cavity enter into the blood stream rapidly, but the immunized mice tend to inhibit this invasion by destruction of injected bacilli. Therefore, if the number of bacilli in blood stream is decided by cultural method at different intervals following infection, the degree of resistance of mice may be observed indirectly.

Infection with 0.01 mg of abortion bacilli is able to produce a bacteremia for 1 week almost at the rate of 100%, but does not kill the mice rapidly.

As shown in table 3, each group of 4 mice was injected with 0.01 mg of abortion bacilli and tail bloods of mice were cultured during the time from half an hour to the 7th day after infection. Then the percentage of the positive cultures was calculated as follows:

$$\frac{\text{Total number of positive cultures in each group}}{\text{Total number of blood cultures in each group}} \times 100$$

(In the case of mice which died within 1 week after infection in the further experiments, the results of blood cultures were estimated to be positive from the date of death to the 7th day).

Thus the lower the percentage of positive cultures the larger becomes resistance of the group of mice against infection.

TABLE 3. Showing the Result of Tail Blood Culture at Various Times after Inoculation of *S. abortus-equi* into Homologously and Heterologously Vaccinated Mice.

MICE IMMUNIZED WITH HEAT-KILLED BACILLI OF	INTERVALS BETWEEN VACCINATION AND INFECTION	RESULTS OF BLOOD CULTURES (Time after Infection)										PERCENTAGE OF POSITIVE CULTURES
		1/2	2	6.5	10	24 hrs.	2	3	5	6	7 days	
<i>S. abortus-equi</i> 0.2 mg	11 days	0	0	0	0	0	+	0	+	0	(+)	6/40 × 100 = 15%
		0	0	(+)	0	0	0	0	0	+	0	
		0	0	0	0	0	0	0	(+)	0	0	
		0	0	0	0	0	0	0	0	0	0	
<i>E. coli</i> 0.2, 0.2	1 day	+	+	0	0	0	0	+	+	0	0	40%
		++	+	0	+	0	+	+	∞	##	##	
		+	0	0	0	0	0	(+)	0	0	0	
		+	0	0	0	0	0	0	0	+	0	
<i>S. abortus-equi</i> 0.2	1 day	##	+	+	+	+	+	+	+	0	0	82.5%
		##	+	(+)	+	(+)	+	+	+	+	+	
		##	##	+	0	+	+	##	∞	∞	D	
		##	+	0	+	0	0	+	+	+	0	
<i>E. coli</i> 0.2	1 day	+	+	0	+	0	0	(+)	0	+	+	80%
		+	+	+	0	(+)	+	+	##	##	##	
		##	+	+	(+)	+	0	+	∞	+	+	
		+	+	0	0	+	(+)	+	+	∞	##	
Controls	—	##	##	+	##	+	+	+	∞	##	##	100%
		##	##	+	+	+	(+)	∞	##	##	##	
		##	##	+	+	+	+	##	##	##	∞	
		##	+	+	+	+	+	+	+	+	(+)	

Challenge dose: 0.01 mg i. p.

0 : negative culture (+) : positive in enrichment culture
 + : 1~50 colonies ++ : 51~100 colonies
 ## : over 100 „ D : death

Calculation of Percentage of Positive Cultures:

$$\frac{\text{Total Number of Positive Cultures in each Group}}{\text{Total Number of Blood Cultures in each Group}} \times 100$$

It may be seen from table 3, that a dose of 0.2 mg of hetero- or homo-vaccine produced slight non-specific resistance, and the increase of the dose to 0.4 mg of hetero-vaccine developed an appreciable non-specific resistance against *S. abortus-equi*. These results seem to indicate that the non-specific intraperitoneal resistance is induced on the 1st day after vaccination depending on the dose of hetero-vaccine.

By means of the above assaying conception, the results of the following experiments have been simplified as is shown in table 4.

TABLE 4. *Non-specific Resistance of Mice which were Tested at Various Intervals between Vaccination and Challenge Dose*

GROUPS IMMUNIZED WITH HEAT-KILLED BACILLI OF	PERCENTAGE OF POSITIVE BLOOD CULTURES (Intervals between Vaccination and Infection)							
	7 hrs.	1	3	6	9	10	10 days	
<i>S. abortus-equi</i>	100%	—	45.4%	—	15.1%	—	—	—
<i>E. coli</i>	96.9%	100%	100%	78.7%	75.7%	100%*	42.7%**	—
Controls	—	—	—	—	—	—	—	100%

Vaccine dose: 0.2 mg i. p.

* This group was injected with 2 doses of vaccine (0.1, 0.1) with a 7-day interval.

** This group was injected with 3 doses of vaccine (0.2, 0.2, 0.2) with 2-day intervals.

Challenge dose: 0.01 mg of *S. abortus-equi* i. p.

Three mice were used in each group.

Percentages of positive cultures in table 4 indicate that 0.2 mg of killed abortion bacilli induced a certain resistance of non-specific type on the 3rd day after vaccination, but the same dose of hetero-vaccine could hardly produce it at any interval. On the other hand, 0.6 mg of hetero-vaccine induced the non-specific resistance on the 10th day after treatment. Results obtained from tables 3 and 4 show that the non-specific resistance caused by a small dose of 0.2 mg of hetero- or homo-vaccine was slightly demonstrated on the 1st day after treatment and increased resistance was developed with a large dose of 0.4 mg of hetero-vaccine at the same interval. Moreover, a considerably larger dose of hetero-vaccine induced the non-specific resistance on the 10th day after treatment. Intraperitoneal treatment with homo-vaccine tends to induce powerfully the non-specific rise in resistance (promunity) in earlier point of time than the specific types of immunity.

Table 5 indicates non-specific resistance caused by various vaccines of hetero-bacteria at different intervals after treatment. As will be noted from this table, heat-killed vaccine of coli did not induce the non-specific resistance 4 hours after treatment, but it sustained the non-specific resistance from the first to the 10th day after treatment.

Hitherto, repeated injections of large vaccine doses were made. However, it is very likely that difference of degree between the non-specific resistance enhanced by the repeated vaccination and the same resistance induced by a single dose may be produced. Therefore, in order to observe the exact effect of large vaccine dose on the rise of non-specific resistance, it is necessary to employ a single injection. In order to avoid the loss of mice with a single injection of large vaccine dose, the killed bacilli should be detoxified. It is well established by Koji ANDO et al. that the toxicity of the killed bacilli is evidently decreased by treatment with alum chromate. Accordingly, formalized coli bacilli were treated with 0.4% alum chromate solution for the decrease of the toxicity. As indicated in table 5,

chrome-vaccine injected repeatedly seems to be as effective on the induction of non-specific resistance as heat-killed vaccine, but formolized vaccine is less effective.

TABLE 5. *Percentage of Positive Blood Cultures of Mice Inoculated with S. abortus-equi at Different Intervals after Vaccination with a Large Dose of Various Vaccines*

VACCINE	DOSE	INTERVALS BETWEEN LAST VACCINATION AND INFECTION	PERCENTAGE OF POSITIVE CULTURES
Heat-killed Bacilli of <i>S. abortus-equi</i>	0.2, 0.2, 0.2 mg	10 days	4.5%
Formol Vaccine of <i>E. coli</i>	"	"	70.4%
Chrome-Vaccine of <i>E. coli</i>	"	"	43.1%
Heat-killed Bacilli of <i>E. coli</i>	"	"	43.1%
"	"	6	47.7%
"	"	1	50.0%
"	0.2, 0.4	4 hrs.	84.1%
"	0.4	"	97.7%
Controls	—	—	79.5%

Interval of repeated vaccination is 2-days.

Challenge dose: 0.01 mg i. p.

Four mice were used in each group.

It may be seen from table 6 that 1 mg of chrome-vaccine injected at a time gives appreciably higher resistance to the mice than is found in the mice group which was given a dose of 0.2 mg. Moreover, as in table 7, in the case of single injection of 1 mg of coli chrome-vaccine, powerful non-specific resistance was observed even 3 weeks after vaccination.

(However, the body weight of the mice used in this experiment was larger than that in the former experiments, so the animals might be much more resistant).

TABLE 6. *Duration of Non-specific Resistance of Mice Vaccinated with Single Small or Large Dose of Chrome-vaccine.*

VACCINE	DOSE (mg)	PERCENTAGE OF POSITIVE BLOOD CULTURES (Intervals between Vaccination and Infection)					
		1	2	4	7	10 days	
Heat-killed Bacilli of <i>S. abortus-equi</i>	0.2	—	—	—	—	38.7%	—
Chrome-vaccine of <i>E. coli</i>	0.2	93.2%	—	95.5%	—	88.7%	—
"	1.0	100.0%	43.5%	63.6%	43.2%	59.1%	—
Controls	—	—	—	—	—	—	90.9%

Challenge dose: 0.01 mg of *S. abortus-equi* i. p.

Four mice were used in each group.

TABLE 7. *Long Duration of Non-specific Resistance of Mice Vaccinated with a Large Dose of Chrome-vaccine*

VACCINE	DOSE (mg)	PERCENTAGE OF POSITIVE BLOOD CULTURES (Intervals between Vaccination and Infection)		
		14	21 days	
Heat-killed Bacilli of <i>S. abortus-equi</i>	0.2	12.1%	—	—
Chrome-vaccine of <i>E. coli</i>	1.0	6.8%	2.3%	—
Controls	—	—	—	90.9%

Challenge dose: 0.01 mg of *S. abortus-equi* i. p.
Four mice were used in each group.

Also other kinds of hetero-vaccine besides coli were used to observe the non-specific rise in resistance against abortion bacilli. As shown in table 8 mice treated with a large dose of different vaccines non-specifically have been found to provide different degrees of resistance.

TABLE 8. *Non-specific Resistance Produced by Vaccination of Various Kinds of Bacteria*

MICE IMMUNIZED WITH HEAT-KILLED BACILLI OF	INTERVALS BETWEEN VACCINATION AND INFECTION	PERCENTAGE OF POSITIVE BLOOD CULTURES
<i>S. abortus-equi</i>	5 days	9.1%
<i>Staphylococcus aureus</i>	"	47.7%
<i>S. senftenberg</i>	"	69.7%
<i>E. coli</i>	"	52.3%
Controls	—	86.4%

Vaccine dose: Three doses (0.25, 0.25, 0.25) were injected at 2-day intervals.
Challenge dose: 0.01 mg of *S. abortus-equi* i. p.

Mice injected with killed abortion bacilli showed non-specific resistance to homologous infection on the 3rd day after vaccination, and also mice injected with a large dose of coli vaccine produced a considerably powerful non-specific resistance to infection with abortion bacilli 10 days after treatment. A single dose of coli chrome-vaccine, such as 1 mg, induced a surprising resistance to infection for 3 weeks under certain conditions. Non-specific resistance on the 1st day after vaccination seemed to be very slight and transient; several hours after treatment it was not observable. However, non-specific resistance induced by heterologous bacteria is weaker compared with the resistance developed by specific vaccination.

CONSIDERATION

According to ØRSKOV and KAUFFMANN, the non-specific resistance is

developed with great rapidity after vaccination and it fades before the appearance of specific immunity. Thus this rapidly produced non-specific resistance was demonstrable during a short time, and they called it "promunity".

On the other hand, PHILIPSON found that the resistance enhanced by means of repeated injections appears more rapidly and is of longer duration than promunity. These workers reported that the non-specific resistance shows the highest degree in the case of injecting both the immunizing and challenge dose by the intraperitoneal route.

The present experiments were designed to determine whether or not the non-specifically enhanced intraperitoneal resistance has effect on the mouse-protection test of equine paratyphoid vaccine. According to the results obtained from the above experiments, in the case of specific immunization, a non-specifically enhanced resistance against infection with abortion bacilli developed more rapidly than specific resistance. Also in the non-specific immunization the same resistance appeared at an early period similar to specific immunization.

Even if this kind of resistance which is observed at an early time may be produced in mouse-protection test of the equine paratyphoid vaccine, it does not practically disturb the test. Therefore, it should be examined whether the non-specific resistance which appeared from early period is continued for a long time or not.

This kind of resistance in non-specific immunization is produced strongly by a large immunizing dose and it seems to last in a constant degree from beginning to the 10th day, even if it is slighter than the resistance in the specific immunization. Under certain conditions, a single injection of 1 mg of heterologous chrome-vaccine demonstrated a considerably powerful resistance to infection with abortion bacilli for 3 weeks, though a slow dissolution of the bacilli might be caused by treatment with alum chromate solution. (It is believed that the decrease of toxicity of the killed bacilli treated with alum chromate solution is due to a slow dissolution of their substances in the tissue). Therefore, it is presumed that mouse-protection test which is performed by employing a large dose of equine paratyphoid vaccine may be influenced by the non-specifically enhanced intraperitoneal resistance.

According to ØRSKOV and KAUFFMANN, and PHILIPSON, regarding the relation of vaccine dose to the non-specific resistance, it was rendered clear that the degree or the duration of this resistance is proportional

to the immunizing dose to some extent. Also the present authors obtained similar results. However, decrease of resistance observed at a very early time after vaccination seems to be due to a toxic effect of a large vaccine dose, as PHILIPSON stated in his report.

The present authors detected the feeble non-specific resistance by culturing the tail blood of mice after infection. PHILIPSON reported that the destruction of intravenously or intraperitoneally injected living bacilli is more rapid in non-specifically immunized mice than in the controls. ØRSKOV demonstrated the increased phagocytosis in the peritoneal cavity of mice immunized non-specifically. Therefore, the blood culture method employed by the present authors seems to be appropriate for observation of the non-specific resistance.

Also other kinds of bacteria than coli, such as staphylococci or *S. senftenberg*, slightly induced non-specific resistance to infection with *S. abortus-equi* 5 days after immunization. This result suggests that the resistance enhanced by coli vaccine must be wholly non-specific.

As indicated in table 4, promunity in specific immunization is more powerful than in non-specific immunization on the 3rd day after vaccination. From this result it may be stated that promunity observed in mouse-protection test of equine paratyphoid vaccine is relatively specific. As to this point, ØRSKOV and KAUFFMANN already indicated that the promunity which appeared in mouse-protection test of typhoid vaccine was not always of complete non-specificity.

Consequently, it is reasonable to consider that non-specifically enhanced intraperitoneal resistance may have interfered with the authors' previous experiment on the mouse-protection test of equine paratyphoid vaccine, which was performed by injecting both immunizing and challenge dose by intraperitoneal route. In order to avoid these confusions in case of mouse-protection test of equine paratyphoid vaccine, injection of vaccine should be made by another route. Kiyoshi ANDO et al. reported that mice injected subcutaneously with killed vaccine of a variant strain of *S. typhimurium* were rendered slightly resistant to intraperitoneal infection with abortion bacilli on the 10th day after vaccination. Nevertheless, from the results obtained by many workers, it is clear that subcutaneous vaccination induces only slightly a non-specific resistance against intraperitoneal infection with homologous or heterologous bacilli, or did not at all. Therefore, when the vaccine dose is very large, it is better to employ subcutaneous injection than intraperitoneal. However, according to previous experiment of the present

authors, subcutaneous treatment did not always give protective power to mice. Thus how to immunize mice most effectively is an important problem for further investigation.

SUMMARY

Mice immunized intraperitoneally with killed vaccine of heterologous bacteria show an enhanced resistance to an intraperitoneal infection with *S. abortus-equi* 1 or 2 days after vaccination and sustain it for a period extending to 10 days. This non-specifically enhanced resistance is inferior to the resistance observed in specific immunization. However, in case of injecting a large dose of heterologous vaccine, non-specific rise in resistance sometimes becomes considerably high and lasts for 3 weeks.

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